(51) International Patent Classification 6 :		(11) International Publication Number: **WO 99/46365**
C12N 5/00, 5/06, 5/08, A61K 38/19, 38/20, 38/21, 49/00, G01N 33/53, 33/567	**A1**	(43) International Publication Date: 16 September 1999 (16.09.99)

(54) Title: METHODS AND COMPOSITIONS FOR THE SELECTIVE EXPANSION OF GAMMA/DELTA T-CELLS

(57) Abstract

The present invention provides a method of increasing the percentage of gamma/delta T-cells in a population of hematolymphoid cells and which gamma/delta T-cells can survive for a prolonged period, comprising: a) contacting a population of hematolymphoid cells with interleukin 12 and a ligand of CD2 which induces responsiveness to interleukin 12; and b) contacting the cells of step (a) with an antibody to CD3 and interleukin-2. The present invention also provides a method of treating cancer, treating an infection, promoting wound healing and enhancing bone marrow engraftment in a subject comprising administering to the subject an effective amount of the cells of this invention.

METHODS AND COMPOSITIONS FOR THE SELECTIVE EXPANSION OF GAMMA/DELTA T-CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for expanding gamma-delta ($\gamma\delta$) T cells in hematolymphoid cell populations for the purpose of generating cell populations enriched for $\gamma\delta$ T cells. Such enriched populations can be used in a variety of applications, including therapeutic uses such as treatment of cancer and infectious diseases, promotion of wound healing and enhancement of bone marrow engraftment, as well as for functional and structural studies of $\gamma\delta$ T cells and their interactions with other components of the immune system.

Background Art

The subset of T cells known as $\gamma\delta$ T cells is normally present in hematolymphoid cell populations as a very small fraction. Although the role of $\gamma\delta$ T cells is not fully understood, data from correlative clinical studies in humans and experimental data from murine models suggest that $\gamma\delta$ T cells may serve to facilitate hematopoietic stem cell (HSC) engraftment in the setting of allogeneic bone marrow transplant (BMT). Current models postulate that $\gamma\delta$ T cells may facilitate engraftment across major histocompatibility complex (MHC) barriers by eliminating or suppressing the function of host-derived cellular elements capable of rejecting donor HSC. There is also evidence to suggest that $\gamma\delta$ T cells can play a beneficial role in the control of infectious disease, in inhibiting tumor growth and in promoting wound healing. In order to employ $\gamma\delta$ T cells at a clinical scale, it would be necessary to isolate or expand $\gamma\delta$ T cells to a significant degree, given their relative infrequency in peripheral blood and other hematological tissues. Isolating $\gamma\delta$ T cells from fresh peripheral blood (PB) or bone marrow (BM) is impractical and prohibitively expensive. Attempts to expand

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γδ T cells *ex vivo* using a variety of standard mitogenic stimuli, including anti-CD3

antibodies or anti-TCR γδ antibodies have yielded γδ T cells which are less functional

in vivo (26). The reason for such decreased function may be related to observations

that, in humans, γδ T cells are extremely sensitive to TCR/CD3 engagement (especially

5   in the presence of IL-2) and undergo apoptosis upon receiving mitogenic stimuli

through the TCR (27). In support of this finding, studies have shown that human γδ T

cell clones readily undergo apoptosis when stimulated simultaneously by anti-

CD3/TCR MAb plus exogenous IL-2 (28,29). This mechanism of activation-induced

cell death (AICD) has not previously been overcome, thereby significantly limiting the

10   usefulness of the γδ T cells expanded as previously described.

Furthermore, enrichment of resting γδ T cells without cytokine and/or mitogen

activation, by isolating this cell fraction from a population of hematolymphoid cells and

pooling the isolated γδ T cells from several populations to increase their number has

15   not proven useful for obtaining γδ T cells in the amounts needed for clinical use.

Specifically, γδ T cells have a very limited life span (about 1-2 weeks) and by the time

a sufficient number of γδ T cells could be isolated and pooled from primary cultures,

most of the cells will have died or would be very near death. Such cells would not be

useful for administration to a subject for the various clinical applications described

20   herein because the γδ T cells would not survive long enough in the subject to facilitate

engraftment, inhibit an infectious process, inhibit tumor growth or promote wound

healing. Thus, what is needed is a method of expanding γδ T cells which can survive

both *ex vivo* for a period of time sufficient to produce sufficiently large numbers of γδ

T cells for clinical use and *in vivo* for a period of time sufficient to impart their

25   intended clinical effect.

The present invention overcomes previous shortcomings in the art by providing

methods for increasing the percentage of γδ T cells, which can survive for prolonged

periods, in a population of hematolymphoid cells and for administering

30   hematolymphoid cell populations which are enriched for these γδ T cells to subjects to

treat cancer, treat infections, promote wound healing and enhance transplant

engraftment. The present invention also provides populations of hematolymphoid cells having increased percentages of γδ T cells which can survive for prolonged periods.

SUMMARY OF THE INVENTION

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The present invention provides a method of increasing the percentage of gamma-delta T cells in a population of hematolymphoid cells and which gamma-delta T cells can survive for a prolonged period, comprising: a) contacting a population of hematolymphoid cells with interleukin 12 and a ligand of CD2 which induces

10   responsiveness to interleukin 12; and b) contacting the cells of step (a) with an antibody to CD3 and interleukin-2.

Also provided is a method of screening a ligand of CD2 for the ability to induce responsiveness to interleukin 12 comprising: a) contacting a population of

15   hematolymphoid cells with the ligand and interleukin 12; b) contacting the cells of step (a) with an antibody to CD3 and interleukin 2; c) maintaining the cells of step (b) in culture for at least seven days; and d) determining the percentage of viable gamma delta T cells in the population of cells of step (c), whereby greater than 10% viable gamma delta T cells identifies a ligand of CD2 having the ability to induce responsiveness to

20   interleukin-12.

Further provided is a population of hematolymphoid cells having greater than 10% gamma delta T cells and which gamma-delta T cells can survive for a prolonged period.

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The present invention also provides a method of treating cancer, treating an infection, promoting wound healing and enhancing bone marrow engraftment in a subject comprising administering to the subject an effective amount of the cells of this invention.

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Various other objectives and advantages of the present invention will become apparent from the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a" or "an" can mean multiples. For example, "a cell" can mean at least one cell or more than one cell.

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The present invention is based on the surprising discovery that the percentage of γδ T cells in a population of hematolymphoid cells can be increased to percentages never before achieved by the administration to the cell population of a particular combination of cytokines and mitogenic stimuli in a specific order. In particular, this

10 invention provides the discovery that γδ T cells can be expanded in a population of hematolymphoid cells by first administering IL-12 and a ligand of CD2, followed by administering IL-2 and T cell mitogenic stimulus. A further unexpected discovery is that the expanded γδ T cells are capable of surviving for a prolonged period of time, making them very useful as source populations for studying the functional and

15 structural aspects of γδ T cells, for identifying additional cytokines and/or other substances which activate γδ T cells and for analyzing their interactions with other immune components, as well as for developing clinical uses for these cells.

Thus, the present invention provides a method of increasing the percentage of

20 gamma-delta T cells in a population of hematolymphoid cells and which gamma-delta T cells can survive for a prolonged period, comprising: a) contacting a population of hematolymphoid cells with interleukin 12 and a ligand of CD2 which induces responsiveness to interleukin 12; and b) contacting the cells of step (a) with a T cell mitogen and interleukin-2. In addition, the method of this invention can further

25 comprise the step of contacting the cells of step (a) with interferon-γ.

That the percentage of γδ T cells in the population of hematolymphoid cells has increased as a result of this method can be determined according to standard methods well known in the art and as described herein for determining percentages of various

30 cell types in a mixed population of cells. For example, the percentage of γδ T cells can be measured in a population of hematolymphoid cells by fluorescence activated cell sorting (FACS) as described in the Examples herein.

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As used herein, γδ T cells "which can survive for a prolonged period" means γδ T cells which can survive under the culture conditions described herein for a period of time which is greater than the period of time during which hematolymphoid cells in

5   primary culture normally survive. For example, a prolonged period of time for survival of γδ T cells means survival of γδ T cells for greater than three weeks and more preferably, for greater than six weeks and most preferably, for greater than eight weeks, under the culture conditions described herein. It is contemplated that the γδ T cells of this invention could be kept alive and functional for the methods described herein

10   indefinitely upon subsequent restimulation of the γδ T cells according to the methods described herein.

Also as used herein, "a ligand of CD2 which induces responsiveness to interleukin 12" means any natural or synthetic molecule, including antibodies to CD2,

15   that, upon interaction with CD2 itself, results in the generation of the cellular and molecular events of signal transduction, thus increasing a cell's responsiveness to IL-12. By responsiveness to IL-12 is meant that the cells which bind the ligand of CD2 are enhanced in their ability to bind and/or respond to IL-12. A ligand of CD2 which can be used in the methods of this invention can include, but is not limited to, an

20   antibody or antibody fragment which specifically binds CD2, CD58, a natural or synthetic homologue of CD58, a receptor-binding fragment of CD58, CD48, a natural or synthetic homologue of CD48 and a receptor-binding fragment of CD48, any of which induce responsiveness to IL-12. A ligand of CD2 can be screened for the ability to induce responsiveness to interleukin-12 according to the methods described herein.

25   For example, the ligand of CD2 can be the monoclonal antibody S5.2 (mouse IgG2a, Becton Dickinson).

It is also appreciated by one of skill in the art that IL-12 and IL-2 can include fragments of IL-12 or IL-2, respectively, which retain the binding and signal

30   transducing activity of an entire IL-12 or IL-2 molecule.

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The T cell mitogen of this invention can be any substance, now known or later identified to have a mitogenic effect on T cells. For example, the T cell mitogen of this invention can be, but is not limited to, an antibody to CD3, pokeweed mitogen, ionomycin, phorbol myristate acetate (PMA), a superantigen (e.g., substances, such as

5   bacterial products, which activate the T cell receptor nonspecifically by binding to less polymorphic domains) and any other T cell mitogen now known or later identified to be a T cell mitogen.

Thus, the present invention further provides a method of screening a ligand of

10   CD2 for the ability to induce responsiveness to interleukin 12 comprising: a) contacting a population of hematolymphoid cells with the ligand and interleukin 12; b) contacting the cells of step (a) with an antibody to CD3 and interleukin 2; c) maintaining the cells of step (b) in culture for at least seven days; and d) determining the percentage of viable gamma delta T cells in the population of cells of step (c), whereby greater than 10%

15   viable gamma delta T cells identifies a ligand of CD2 having the ability to induce responsiveness to interleukin-12. The screening method of this invention can further comprise the step of contacting the cells of step (a) with interferon gamma. The percentage of viable γδ T cells can be determined according to the methods provided in the Examples herein and as are well known in the art. For example, the γδ T cells can

20   be selectively separated from other cell types by FACS, as described herein and the viability of the γδ T cells can be determined by trypan blue staining, as also described herein.

As used herein, "hematolymphoid cells" means any cells derived from bone

25   marrow precursor cells, which comprise myeloid cells, erythroid cells, lymphoid cells, platelets and the like, as is well known in the art. The hematolymphoid cells which can be used in the methods of this invention can be bone marrow cells, peripheral blood mononuclear cells and/or cord blood cells, as well as cells from any tissue in which γδ T cells and/or their precursors can be found (e.g., skin, intestinal epithelium, thymus,

30   liver, spleen, fetal tissues such as fetal liver and fetal thymus). Furthermore, the hematolymphoid cells of this method can be from any animal which produces γδ T cells, which can be any mammal and in a preferred embodiment, is a human.

Furthermore, the hematolymphoid cells used in the methods of this invention can be cells which are removed from a subject and returned to the subject (autologous). Additionally, the hematolymphoid cells can be removed from a donor and administered

5   to a recipient of the same species, that is a genetically different (allogeneic); removed from a donor and administered to a recipient that is genetically identical (syngeneic), and/or removed from a donor and administered to a recipient of a different species (xenogeneic).

10   The subject, donor and/or recipient of the present invention can be diagnosed with cancer (e.g, leukemia, lymphoma and solid tumors) tissue injury (e.g., due to trauma, burns, graft-versus-host disease or autoimmune destructive processes) and/or an infection by a pathogen (e.g., human immunodeficiency virus or other pathogenic virus, pathogenic bacteria, parasites, mycoplasma, pathogenic fungi, etc.).

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In addition, the hematolymphoid cells used in the methods of this invention can be removed from a donor and administered to a transplant recipient. The transplant recipient can be diagnosed with cancer, tissue injury and/or an infection by a pathogen. Thus, the cells of this invention can be used to enhance engraftment of the recipient's

20   transplanted tissue and/or treat the recipient's cancer, infection and/or tissue injury.

The population of hematolymphoid cells in which the percentage of γδ T cells is increased by the methods described herein can be further enriched for γδ T cells to yield a population of hematolymphoid cells having any percentage of γδ T cells, up to

25   100%γδ T cells. Such enriched cell populations can be administered, in a pharmaceutically acceptable carrier, to a subject for a variety of therapeutic treatments, as described herein. Thus, the method of increasing the percentage of γδ T cells in a population of hematolymphoid cells as described herein can also comprise the step of further increasing the percentage of γδ T cells in the population of hematolymphoid

30   cells of step (c) of the above-described method by procedures well known in the art and as described herein, such as selective separation of γδ T cells by fluorescence activated cell sorting (FACS), affinity column chromatography, immunomagnetic separation,

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density gradient centrifugation and cellular panning, as well as any other method for selectively separating cell subpopulations, as would be well known to an artisan. In a preferred embodiment, the γδ T cells are selectively separated by FACS with negative staining methods, as described in the Example section herein, to avoid stimulation of

5   the γδ T cells.

The present invention also provides a population of hematolymphoid cells having greater than 10% γδ T cells and which γδ T cells s can survive for a prolonged period, as defined herein. The population of hematolymphoid cells of this invention

10   can have greater than 20%γδ T cells, greater than 30%γδ T cells, greater than 40% γδ T cells, greater than 50%γδ T cells, greater than 60% γδ T cells, greater than 70% γδ T cells, greater than 80% γδ T cells and greater than 90% γδ T cells and can have any percentage of γδ T cells up to 100% γδ T cells.

15   The hematolymphoid cells having the percentages of γδ T cells listed above can be bone marrow cells, peripheral blood mononuclear cells and/or cord blood cells, as well as cells from any tissue in which γδ T cells and/or their precursors can be found, as described herein. Thus, typically the other cell types included in the population of hematolymphoid cells can include but are not limited to, red blood cells, platelets,

20   white blood cells (e.g., neutrophils, eosinophils, basophils, monocytes, lymphocytes), tissue cells (e.g, macrophages, epithelial cells, endothelial cells, dendritic cells, mast cells) and the like as would be well known to an artisan. Furthermore, the hematolymphoid cells of this invention can be from any animal which produces γδ T cells, which can be any mammal and in a preferred embodiment is a human.

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As described above, the cells of this invention can be administered to a subject to treat various disorders as well as to enhance transplant engraftment. Thus, the present invention provides a method of treating cancer in a subject comprising administering to a subject diagnosed with cancer an effective amount of the cells of this

30   invention. The cancer of this invention can be any malignant blood disorder, acute and/or chronic leukemia, multiple myeloma, lymphoma, solid tumors (e.g., breast cancer, pancreatic cancer). In addition, the cells of this invention can be administered

to a subject to treat aplastic anemia, bone marrow failure and any other hematological disorder and/or immunological disorder which can be treated by administration of the cells of this invention.

5   Further provided is a method of treating an infection in a subject comprising administering to a subject diagnosed with an infection an effective amount of the cells of this invention.

A method of promoting wound healing in a subject is also provided, comprising
10   administering to a subject having a wound an effective amount of the cells of this invention.

In addition, a method of enhancing bone marrow engraftment in a bone marrow recipient is provided, comprising administering to the recipient an effective amount of
15   the cells of this invention.

The hematolymphoid cells of this invention can be removed from a subject or donor and treated according to the methods described herein to increase the percentage of γδ T cells in the hematolymphoid cell population and administered to the same
20   subject or to a recipient by *ex vivo* methods for removing cells, maintaining the cells in culture and re-administering them, as well known in the art. Standard methods are known for removal of cells from a subject or donor (e.g., phlebotomy, apheresis) and infusion of cells into a subject or recipient.

25   It is also contemplated that the percentage of γδ T cells can be increased in a population of hematolymphoid cells *in vivo*. For *in vivo* methods, the ligand of CD2, the T cell mitogen, IL-12 and IL-2 can be administered to the subject in a pharmaceutically acceptable carrier. For example, an anti-CD2 antibody which induces responsiveness to IL-12 (e.g., MAb S5.2) can be administered intravenously to a
30   subject in a dosage range of 0.01 to 10 mg/kg body weight; IL-12 can be administered intravenously to a subject in a dosage range of 100 to 1000 nanograms/kg of body weight (41); an anti-CD3 antibody (e.g., OKT3) can be administered intravenously to a

subject in a dosage range of 1-10 mg (manufacturer's package insert and Physician's

Desk Reference, latest edition); and IL-2 can be administered intravenously to a subject

in a range of 200,000 to 800,000 international units (IU) (manufacturer's package insert

and Physician's Desk Reference, latest edition). The exact amount of these substances

5   can vary from subject to subject, depending on the species, age, weight and general

condition of the subject, the severity of the disorder being treated, the particular

substance being used, its mode of administration and the like. Thus, it is not possible

to specify an exact amount for every substance in every subject. However, an

appropriate amount can be determined by one of ordinary skill in the art using only

10   routine experimentation given the teachings herein (see, e.g., *Remington's*

Pharmaceutical Sciences).

As an example, on Day 0, 0.1 mg/kg of anti-CD2 and 500 nanogram/kg of IL-

12 is administered in a pharmaceutically acceptable carrier as an intravenous infusion

15   to the subject. On Day 1, 600,000 IU of IL-2 is administered in a pharmaceutically

acceptable carrier as a 15 minute intravenous infusion and 5 mg of anti-CD3 as a single

dose is administered in a pharmaceutically acceptable carrier, intravenously as a rapid

injection. To determine the efficacy of administration of these substances, the

peripheral blood of the subject can be analyzed by FACS before and after

20   administration to determine if the percentage of $\gamma\delta$ T cells has increased. The time

intervals for such measurement of the peripheral blood can be hours, days, weeks

and/or months after administration of the substances listed herein. Other clinical

parameters which can be monitored to determined the efficacy of administration of

these substances can include measurement of 1) disease progression or response, 2) rate

25   of graft failure, 3) graft-versus-host disease assessment, 4) time to engraftment after

bone marrow transplant, 5) rate of infectious complications and/or 6) objective

parameters of epithelial mucosal tissue injury, as would be known to one of skill in the

art.

30   Thus, the present invention further provides a method for 1) treating cancer in a

subject; 2) treating an infection in a subject; 3) promoting wound healing in a subject;

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and/or 4) enhancing engraftment of a transplant in a subject by increasing the percentage of γδ T cells in the subject comprising:

   a) administering to the subject on day 0 an effective amount of a ligand of CD2 which induces responsiveness to IL-12, in a pharmaceutically acceptable carrier and an

5   effective amount of IL-12; and

   b) administering to the subject on day 1 an effective amount of a T cell mitogen in a pharmaceutically acceptable carrier and an effective amount of IL-2 in a pharmaceutically acceptable carrier, whereby the administration of the substances of steps (a) and (b) increases the percentage of γδ T cells in the subject.

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   As described above, the cells and/or substances administered to a subject *in vivo* can be in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the cells or substances, without causing any

15   undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

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   The cells of the present invention and the substances which increase the percentage of γδ T cells *in vivo* are typically administered parenterally and are most typically administered by intravenous injection, although other parenteral routes of administration, such as intramuscular, intradermal, subcutaneous, intraperitoneal

25   administration, etc., is also contemplated. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained. See, e.g., U.S.

30   Patent No. 3,710,795, which is incorporated by reference herein.

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The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

5   **EXAMPLES**

EXAMPLE I

Isolation of PBMC, adherent cell-depleted PBMC and CD14$^+$ monocytes.
PBMC, replete with monocytes, were isolated from healthy, human volunteers by
10   Ficoll gradient centrifugation of peripheral blood anticoagulated with heparin. PBMC
were depleted of monocytes by removal of plastic-adherent cells, as previously
described (15). CD14+ monocytes were purified directly from PBMC by sorting FITC-
CD14 (Leu-M3)$^+$ cells, as described below.

15   *Generation and maintenance of cell cultures.* Cultures of PBMC or
monocyte-depleted PBMC were initiated at a cell density of 1 x 10^6 cells/mL in 24-well
flat-bottom tissue culture trays (Costar, Cambridge, MA) and were maintained in 5 %
CO_2 at 37° C in complete medium consisting of RPMI-1640 (Applied Scientific, San
Francisco, CA), 10% autologous human plasma, 2 mM L-glutamine, 100 U/mL
20   penicillin, 100 U/mL streptomycin and 50 µM 2-ME (GIBCO, Grand Island, NY). On
the day of culture initiation, human rIFN-γ (Boehringer Mannheim, Indianapolis, IN)
was added at a concentration of 1,000 U/mL. 24 hours later, cultures were stimulated
by the addition of 10 ng/mL anti-CD3 MAb (Muromonab-CD3, Orthoclone OKT3,
Orthobiotec, Raritan, NJ) and 300 U/mL rIL-2 (Boehringer Mannheim, Indianapolis,
25   IN).

Commercially prepared recombinant IL-12 (rIL-12) (Genetics Institute,
Cambridge, MA) was used at a final concentration of 10 U/mL and was added as a
single dose to appropriate cultures on the day of initiation. A neutralizing polyclonal
30   anti-IL-12 antibody, a neutralizing monoclonal anti-IL-12 antibody, or an irrelevant
isotype control antibody (R&D Systems, Minneapolis, MN) were used at
concentrations of 20 µg/mL and were added as a single dose to cultures on the day of

initiation. Cell density in all cultures was maintained at 1 to 2 x 10^6 cells/mL, with the addition of fresh media and transferred to larger tissue culture flasks as required. Fresh complete medium with 300 U/mL IL-2 was added to cultures every 5 days.

5   *Analysis and cell sorting by flow cytometry.* Cells were stained using FITC- or PE-directly conjugated MAbs recognizing CD3, CD14, CD19, CD20 or CD56 (Becton-Dickinson, San Jose, CA). Directly-conjugated isotype-matched irrelevant antibodies served as controls. Cells were stained for 30 min at 4° C in staining buffer consisting of Hank's buffered saline solution (HBSS, Mediatech, Herndon, VA) containing 2%

10   autologous human plasma. Excess Ab was removed by dilution with 10 volumes of staining buffer, followed by centrifugation at 500x g. Stained cells were immediately analyzed using a FACSCAN flow cytometer (BDIS, San Jose, CA), or sterile-sorted using a FACSVANTAGE cell sorter (BDIS, San Jose, CA). List-mode data were acquired using forward- and side-scatter gates appropriate for viable lymphoid cells or

15   monocytes. Data analysis was performed using CellQuest™ or LYSIS-II software (BDIS, San Jose, CA).

          Detection of IL-12 by ELISA. Monocytes, B lymphocytes, T lymphocytes and NK cells were isolated directly from fresh PBMC by sorting the respective $CD19^-$

20       $CD14^+$ $CD14^-CD19^+$, $CD3^+CD56^-$ and $CD3^-CD56^+$ cell populations. Equivalent numbers (1 x 10^5) of each sorted population were cultured separately as described above, with or without the addition of IFN-γ on the day of culture initiation. Supernatants were harvested after 72 hours, centrifuged to remove cellular debris and stored at -20° C. Detection of IL-12 in culture supernatants was performed using an

25   enzyme-linked immunoassay following the manufacturer's instructions (IL-12 Quantikine™ Assay, R&D Systems, Minneapolis, MN).

          Upon phenotypic analysis of cultures established as described above, it became apparent that inclusion of one-anti-CD2 mAb (clone S5.2, mouse IgG2a, Becton

30   Dickinson) resulted in over-representation of γδ T cells in short-term cultures as detected by FACS. At 14 days, cultures established as above (with or without anti-CD2 MAb S5.2) were analyzed for percentage of T cells using FITC-conjugated MAb

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to γδ TCR, gating on propidium iodide (PI) negative populations of lymphoid cells. Cultures established without addition of IL-12, to which a single dose of anti-CD2 MAb S5.2 was added at 10 mcg/mL, were found to contain a significant percentage of γδ T cells (24%). In contrast, isotype control antibody for S5.2 (mouse IgG2a, Becton

5   Dickinson) resulted in no increase in percentage of γδ T cells.

Anti-CD2 MAb-induced γδ T cell proliferation was augmented by the addition of exogenous rIL-12. If both anti-CD2 MAb and rIL-12 (10 U/mL) were included at the initiation of cultures, an even greater percentage of γδ T cells was detected after 14

10   days in culture (32%). Addition of IL-12 with only the isotype control for anti-CD2 resulted in a minimal increase of γδ T cell percentage. If anti-CD2 MAb was added to culture but neutralizing MAb to IL-12 was also added at the initiation of culture, γδ T cell proliferation was completely inhibited, indicating that the CD2-mediated γδ T cell proliferation is dependent upon the presence of endogenous IL-12 in these cultures.

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Endogenous IL-12 exerts an important effect upon γδ T cell expansion in this culture system. Monocytes are an important cellular source of IL-12 and interferon-gamma (IFNγ) stimulates the production of IL-12 by monocytes. Though IFNγ plays a role in the stimulation of IL-12 production in these conditions, it is also possible that

20   IFNγ induces the expression of other soluble factors or surface structures which may also contribute to the observed γδ T cells expansion.

An extensive literature exists describing the role of various anti-CD2 mAbs to engage various epitopes of CD2 and the resulting proliferation of various T cells

25   subsets, including γδ T cells (11,13,15). In addition to S5.2, several other anti-CD2 MAbs (and their corresponding isotype controls) were assessed for the ability to induce γδ T cell expansion under the culture conditions described, now incorporating the addition of exogenous IL-12 at the time of initiation (day 0: IFNγ, 1000 u/mL and Il-12, 10 U/mL; day 1: OKT-3, 10 ng/mL and IL-2, 300 U/mL). Antibodies to be tested

30   were added to cultures at the time of initiation (day 0) at equivalent concentrations (5 mcg/mL). These antibodies included 6F10.3 (mouse IgG1, Immunotech); 39C1.5 (rat IgG2a, Immunotech) and LT-2 (mouse IgG2b, Serotech). None of these reagents tested

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was comparable to S5.2 in ability to significantly induce γδ T cell expansion. These
data suggest that anti-CD2 MAb S5.2 engages a particular CD2 epitope not engaged by
the other CD2 MAbs, resulting in the observed effect, or alternatively, S5.2 is more
effectively cross-linked by FcR-bearing cells present in the heterogeneous PBMC

5   cultures, resulting in more efficient signaling via CD2. Anti-CD2 MAb S5.2 is a
commercially available preparation, available in a low endotoxin azide-free form.

        Cultures of fresh PBMC were initiated as described, receiving IFNγ on day O
and OKT3 and IL-2 on day 1. In addition, on day 0, IL-12 (10 U/mL) or PBS was

10   added as was an amount of MAb S5.2 (0, 1, 5 and 10 mcg/mL). After 14 days in
culture, absolute cell numbers of both γδ T cells and αβ T cells were calculated from
total cell numbers present in cultures and the percentage of cells in culture of the
corresponding phenotype as determined by FACS. Data were expressed as fold
expansion of T cells over day 0 cell numbers for αβ T cells or γδT cells. Data from

15   these experiments demonstrated that addition of exogenous IL-12 without the addition
of anti-CD2 MAb S5.2 resulted in only a marginal augmentation of γδ T cells
(expansion 40 fold to 60 fold), which was minimal compared to the expansion elicited
by engaging CD2 in the presence of IL-12 (over 200 fold). Also, addition of this
combination appears to have a more pronounced effect on γδ T cells as compared to αβ

20   T cells.

        These studies show that engagement of CD2 is critical for expansion of γδ T
cells from these cultures. This expansion requires the presence of IL-12, whether
provided exogenously or produced endogenously. Cultures of PBMC were stimulated

25   with IFNγ on day O, followed by OKT3 and IL-2 on day 1. In cultures to which
neither IL-12 nor anti-CD2 MAb S5.2 were added, γδ T cells expanded only 38 fold.
In identical cultures receiving anti-D2 MAb S5.2 (5 mcg/mL) on day 0, γδ T cells were
expanded 83 fold, far less than the 229 fold expansion observed if both MAb S5.2 and
IL-12 (10 U/mL) were included. Thus, endogenously produced IL-12, while less

30   pronounced in its ability to stimulate γδ T cell expansion (presumably related to its less
than pharmacological concentration present in culture) is still clearly important, as
indicated by loss of γδ T cell expansion on adding neutralizing monoclonal anti-human

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IL-12 antibody (25 mcg/mL, R&D Systems). Isotype control for anti-human IL-12
antibody had no effect.

Assessment of γδ T cell expansion in purified sorted populations:

5   **Measurements of proliferation.** Data derived in clones showed that some anti-CD2
MAbS can preferentially stimulate proliferation of γδ T over αβ T cells. The
proliferative capacities of γδ T cells and αβ T cells sorted to high purity from identical
cultures of fresh PBMC stimulated as described herein, were compared. [^{3}H]thymidine
incorporation and cell survival (as determined by cell counting) were examined in order

10   to determine if γδ T cells had a proliferative advantage over αβ T cells, or possibly
surviving better, thus accounting for their eventual over-representation in cultures.

Sorting of αβ T cells and γδ T cells by negative selection. In order to avoid
undesirable stimulation which might occur if γδ T cells were sorted using either anti-

15   TCRγδ or anti-CD3 antibodies, γδ T cells were isolated by negative sorting. Thus, γδ
T cells were identified and sorted on the basis of being TCRαβ- and CD5+. (Greater
than 96% of CD3+ cells also stained for CD5). All cells sorted were taken from the PI-
gate to assure viability. Briefly, γδ T cells were stained using FITC- or PE-directly
conjugated MAbS recognizing CD5 and TCRαβ (Becton-Dickinson, San Jose, CA).

20   Directly-conjugated isotype-matched irrelevant antibodies served as controls. Cells
were stained for 30 min at 4° C in staining buffer consisting of Hank's buffered saline
solution containing 3% autologous human plasma. Excess Ab was removed by dilution
with 10 volumes of staining buffer, followed by centrifugation at 500x g. Stained cells
were immediately sorted using a FACSVANTAGE cell sorter (BDIS, San Jose, CA)

25   equipped with a high-speed (Turbo-Sort) sort option. Data analysis was performed
using CellQuest™ software (BDIS, San Jose, CA). In a similar manner, αβ T cells
were sorted as TCRγδ- and CD5+.

γδ T cells sorted from 3 week old short-term cultures (initiated with mAb

30   **S5.2) proliferate to a greater degree than αβ T cells.** Cultures of human PBMC were
initiated as described above, receiving on day 0: IFN-γ, IL-12 and anti-CD2 mAb S5.2.
OKT3 and IL-2 were added on day 1. Cultures were maintained at a cell density of 1-2

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x 10^6 cells/mL for 3 weeks with the addition of fresh medium and IL-2 (100 U/mL) as
needed. On day 21, highly pure γδ T cell and αβ T cell populations were sorted from
these initial cultures by negative selection as described above and were replated at
equivalent cell density (5,000 cells/well) in 96-well micro-titer trays, receiving either

5   IL-2 at 100 U/mL or nothing. After 24 hours, sorted cells were labeled and harvested
as above. In these cultures, γδ T cells proliferated to a greater extent in response to IL-
2 when compared to αβ T cells arising from identical cultures, in contrast to the above
data obtained in fresh PBMC cultures.

10   Because γδ T cells have been reported to be highly sensitive to TCR/CD3
engagement (especially in the presence of IL-2) and undergo apoptosis upon receiving
mitogenic stimuli through the TCR (10), an interpretation of these data is that γδ T
cells found in these longer-term cultures represent the outgrowth of a subset of cells
which upon engagement of CD2 were imparted a survival advantage, i.e., became

15 resistant to apoptosis or activation-induced cell death (AICD) caused by engagement of
TCR/CD3.

        Total cell numbers. To investigate whether γδ T cells present in longer-term
cultures represented the out-growth of a population of cells more resistant to apoptosis

20 (either through inherent or acquired means), the following experiment was performed.
PBMC were stimulated as described with IFN-γ, IL-12 and MAb S5.2 on day 0,
followed by OKT3 and IL-2 on day 1. Cultures were maintained at a cell density of 1-2
x 10^6 cells/mL for 3 weeks with the addition of fresh media and IL-2 (100 U/mL) as
needed. On day 21, highly pure γδ T cell populations and αβT cell populations were

25 sorted from these initial cultures by negative selection as described above. 150,000
highly purified cells of each sorted population were placed separately in 2 mLs of
complete RPMI containing 100 U/mL IL-2. Cells were stimulated with nothing
(indicated as "no addition"); IL-12 alone; plastic-immobilized anti-CD2 MAb S5.2
alone; or both IL-12 and plastic-immobilized anti-CD2 MAb S5.2. Cells were kept at

30 37°C in 5% CO_2 for one week at which point they were counted and scored as live
using trypan blue, which was confirmed with acridine orange assessment using a
fluorescence microscope. These data demonstrated that, whereas αβ T cells survived

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very poorly and responded to no signals, in contrast, greater numbers of γδ T cells survived that were stimulated with nothing. These data also demonstrated that engagement of CD2, but only in the presence of IL-12, imparted an even greater survival advantage on γδ T cells, whereas IL-12 alone or MAb S5.2 alone did not.

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In conjunction with the data obtained from the [³H]thymidine incorporation studies described herein, these findings confirm that some γδ T cells persist in S5.2-initiated cultures, surviving to a greater extent than αβ T cells. These data also have important *in vivo* immunotherapeutic implications. If a subset of γδ T cells is selected

10   to survive by an initial encounter *in vitro* with a "death-rescue signal" (CD2 engagement in the presence of IL-12) and if the "death rescue signal" can again impart a survival advantage at a later time, then engagement of the natural ligand for CD2 (CD58/LFA-3) in the presence of IL-12 *in vivo* can also serve to preserve cell viability and/or function.

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Human T cells are thought to undergo apoptosis upon engagement of their TCR/CD3 complex. This process has been termed activation-induced cell death (AICD) and likely plays an important role in regulating T cell proliferative responses to mitogenic stimuli both *in vitro* and *in vivo*. The mechanisms by which AICD occurs are

20   not entirely understood, although it has been shown that in mature, previously stimulated T cells, initiation of programmed cell death (apoptosis) likely involves the transduction of death-initiating signals generated by engagement of the Fas (CD95/Apo-1) antigen present on T cells and that apoptosis triggered by TCR/CD3 signaling is not restricted to CD4+CD8+ immature thymocytes or transformed

25   leukemic T cell lines but can also occur in IL-2-dependent normal γδ T cells (10,19-21). It has also been shown that Fas/CD95 engagement and subsequent mobilization of intracellular Ca^{2+} are causally related to apoptosis occurring in human γδ T cells clones (22). In light of these observations, studies were conducted to determine if CD2 engagement in the presence of IL-12 provides a "protective" signal to a subset of γδ T

30   cells which rescues them from AICD caused by mitogenic OKT3 and IL-2 and to determine if these "AICD-protected" cells are the γδ T cells which eventually come to be "over-represented" in S5.2-stimulated cultures. Studies were also conducted to

prospectively identify which γδ T cells might be receiving protective signals from CD2
engagement in the presence of IL-12.

Flow cytometry. Initial observations were made using bulk T cell cultures and
5   not T cell clones or T cell lines. Therefore, the issue of apoptosis was examined using
a flow cytometric approach. Flow cytometry using a four-color dual laser configuration
(FACS Calibur flow cytometer or FACS Vantage cell sorter, Becton Dickinson) allows
a discrete population of cells (such as γδ T cells) to first be defined by surface
phenotype from within a heterogeneous population of cells. Simultaneously,
10   biologically relevant processes related to activation, proliferation, cytokine production
or apoptosis can be examined, provided the proper reagents and methods are employed.
Annexin V conjugated to FITC has been used to detect apoptosis in a variety of cell
types. Annexin V binds with high affinity to phosphatidylserine which is normally
confined to the inner plasma membrane leaflet of live, non-apoptotic cells.
15   Phosphatidylserine externalization is an early and widespread event associated with
apoptosis in a variety of human cell types, regardless of the initiating stimulus. Thus, in
combination with fluorescent-conjugated antibodies, Annexin V-FITC can be used to
examine apoptosis in a phenotypically defined subpopulation of cells in heterogeneous
cell cultures. Furthermore, by utilizing propidium iodide (PI) as an indicator of cell
20   viability, viable cells (Annexin$^-$/PI$^-$) can be distinguished both from apoptotic cells
(Annexin$^+$/PI$^-$) and from necrotic cells (Annexin$^+$/PI$^+$).

1. γδ T cells at rest are more prone to apoptosis than αβ T cells. T cell
subsets from fresh PBMC isolated from several different individuals were first
25   characterized for their "baseline" apoptotic tendencies. γδ- and αβ T cells were first
identified and gated on the basis of surface staining with directly conjugated antibodies
recognizing CD3 and TCRγδ. Gated populations of γδ- and αβ T cell populations
were then analyzed with respect to Annexin-FITC and PI. Statistics were expressed as
the percentage of cells appearing in the corresponding dot-plot quadrants. Viable,
30   apoptotic and necrotic regions were defined using the Jurkat T cell line treated with the
anti-Fas (CD95/Apo-1) monoclonal antibody CH-11 (Kamiya Biomedical) or isotype
control IgM.

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These data revealed that, whereas 89% of αβ T cells were found to be "viable" (Annexin⁻/PI⁻), only 59% of γδ T cells were found to be "viable", that is are not actively undergoing apoptosis, as measured by Annexin V-FITC binding. These cells were all isolated, processed and analyzed in the exact same manner, minimizing the

5   possibility that an artifact associated with preparation alone accounts for these findings. Furthermore, experiments were performed using various combinations of Annexin V-FITC and -PE as well as anti-CD3, anti-TCRγδ and anti-TCRαβ reagents conjugated to alternate fluorophores to address the possibility that compensation artifacts alone account for these findings. Results of several separate experiments were found to be

10   comparable.

2. Immediate effects of mitogenic and "protective" signals on αβ-and γδ T cell apoptosis: Measurement of AICD within discrete subpopulations of T cells in bulk cultures after 18 hours.

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"Rescue" and "Death" signals. The above data suggest a slightly greater susceptibility to apoptosis in resting γδ T cells as compared to αβ T cells. Studies were also conducted to determine whether γδ T cells display a greater susceptibility to apoptosis upon receiving mitogenic (AICD) signals and whether CD2 engagement in

20   the presence of IL-12 could serve as a "rescue signal" from AICD caused in γδ T cells. For the purposes of this study, the following combination was employed as the "standard" method of generating S5.2-stimulated γδ T cells. On **day 0**, fresh PBMC in complete RPMI containing 10% autologous human plasma at a density of 1 x 10⁶ cell/mL received: IFN-γ (1,000 U/mL); IL-12 (10 U/mL) and anti-CD2 MAb S5.2 (5

25   mcg/mL). On **day 1** cultures received: OKT3 (10 ng/mL) and IL-2 (300 U/mL). Furthermore, the day 0 stimuli were designated as "rescue" signals and day 1 stimuli (a standard mitogenic combination) were designated as the "death" signals.

PBMC were prepared fresh from peripheral blood by Ficoll density

30   centrifugation. Cultures were initiated receiving either day 1 "death" signals alone; day 0 "rescue" signals alone or both day 0 + day 1 signals, i.e., "standard" conditions. At 18 hours after receiving the day 1 signals (mitogen), cultures were analyzed for

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apoptosis by four-color flow cytometry as described above. γδ- and αβ T cell populations from within each PBMC culture were first identified and gated in two colors (CD3-APC and TCRγδ-PE). Statistics were expressed as the percentage of cells appearing in the corresponding dot-plot quadrants: viable (Annexin⁻/PI⁻), apoptotic

5   (Annexin⁺/PI⁻).

These results demonstrated that γδ T cells were highly sensitive to AICD in response to day 1 ("death") signals alone. As stated previously, close to 60 % of resting γδ T cells were viable (Annexin⁻/PI⁻). However, after receiving mitogenic stimuli, this

10   percentage falls to 5%. In contrast, αβ T cells in the same cultures demonstrated no such sensitivity to these mitogenic conditions, with close to 90% of these cells remaining viable.

Furthermore, whereas only 5% of γδ T cells were found to be viable after

15   receiving day 1 "death" signals alone, close to 20% of γδ T cells were found to be viable, if, in addition to receiving the same day 1 "death" signals, they received day 0 "rescue" signals. The greatest proportion of viable γδ T cells was found in cultures initiated with "rescue" signals alone, i.e., those receiving no mitogenic stimulation via OKT3 and IL-2. However, in these cultures, significant T cell expansion (either αβ or

20   γδ) did not occur even after two weeks, as assessed by gross cell numbers. Apoptosis induced in αβ T cells under the same conditions was negligibly affected by either "death" or "rescue" signals. In this regard, αβ T cells serve as a control, indicating that it is the γδ T cell compartment which is most affected by these manipulations. These finding taken together, demonstrate that CD2 engagement in the presence of IL-

25   12 can provide a "protective" signal to a subset of γδ T cells which eventually arises as the dominant population in these cultures.

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3. Long-term effects of mitogenic and "protective" signals on αβ-and γδ T cell apoptosis: Measurement of AICD within discrete subpopulations of T cells in bulk cultures after 7 days.

5   Similar experiments described above were performed, but cultures were maintained for longer periods, to determine if CD2 engagement in the presence of IL12 provides a "protective" signal which allows for an apoptosis resistant population of γδ T cells to dominate the culture. PBMC were prepared fresh from peripheral blood by Ficoll density centrifugation. Cultures were initiated, receiving either day 1 "death" signals alone or both day 0 + day 1 signals, i.e., "standard" conditions. Cultures receiving no mitogenic stimulation via OKT3 and IL-2 ("rescue" signals alone) failed to proliferate. One week after receiving the day 1 signals (mitogen), cultures were analyzed for apoptosis by four-color flow cytometry as described above.

15   In cultures initiated with both rescue signals and mitogenic stimuli, over 80% γδ T cells were found to be viable after one week, a significantly larger percentage than that found at rest (less than 60 %). In contrast, in cultures stimulated without the rescue signals and receiving only the mitogenic signals, only approximately 50% of γδ T cells were found to be viable. These data appear to indicate that CD2 engagement in the presence of IL-12 is indeed providing a "protective" signal to a small subset of γδ T cells, these cells possibly becoming the dominant population in cultures by virtue of an acquired resistance to AICD. These cells would be expected to be less susceptible to apoptosis, consistent with the Annexin/PI data from 18 h cultures, as described above.

25   The cell cultures described herein which have been treated by standard conditions and which have been shown to contain up to 80% viable γδ T cells after one week, have been maintained in culture for a time period exceeding eight weeks. These cultures can be perpetuated indefinitely by cell passage and addition of fresh medium as needed and restimulation of the cells by administering the substances described herein for standard conditions at regular intervals, as can be determined by routine methods.

EXAMPLE II

Isolation of PBMC and adherent cell-depleted PBMC. Peripheral blood was obtained by phlebotomy of normal, healthy human volunteers. All samples were collected into tubes anticoagulated with heparin. PBMC were isolated by Ficoll
5 gradient centrifugation of whole blood. PBMC were depleted of monocytes by removal of plastic-adherent cells, as previously described (15). Monocyte depletion was confirmed by FACS prior to use in cultures.

Generation and maintenance of cell cultures. Cultures of PBMC were initiated
10 at a cell density of 1 x 10^6 cells/mL in 24-well flat-bottom tissue culture trays (Costar, Cambridge, MA) and were maintained in 5 % CO_2 at 37° C in complete medium consisting of RPMI-1640 (Mediatech, Herdnon, VA), 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 50 μM 2-ME (GIBCO, Grand Island, NY). On the day of culture
15 initiation (day 0), human rIFN-γ (1,000 U/mL, Boehringer Mannheim, Indianapolis, IN); human rIL-12 (10 U/mL, R&D Systems, Minneapolis, MN) and mouse anti-human CD2 MAb clone S5.2 (1-10 μg/mL, mouse $IgG2_a$, Camfolio, Becton Dickinson, San Jose, CA) were added. 24 hours later (day 1), cultures were stimulated by the addition of 10 ng/mL anti-CD3 MAb OKT3 (mouse $IgG2_a$, Orthobiotec, Raritan, NJ)
20 and 300 U/mL rIL-2 (Boehringer Mannheim, Indianapolis, IN). Where indicated, neutralizing monoclonal anti-human IL-12 antibody, or an irrelevant isotype control antibody (R&D Systems, Minneapolis, MN) were added as a single dose at a final concentration of 25 μg/mL on the day of culture initiation. Neutralizing monoclonal anti-human CD58 MAb clone L306 (mouse $IgG2_a$, Camfolio, Becton Dickinson, San
25 Jose, CA) or $IgG2_a$ isotype control antibody were added to cultures where indicated, at a final concentration of 5 μg/mL. In all cultures, cell density was maintained at 1 to 2 x 10^6 cells/mL, with the addition of fresh media and transfer to larger tissue culture flasks as required. Fresh, complete medium with 10 U/mL IL-2 was added to cultures every 7 days.
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Immobilization of stimulatory antibodies. When used in an immobilized form, MAbs OKT3 and anti-human CD2 MAb clone S5.2 were bound to plastic tissue culture

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plates as previously described (30). Briefly, MAbs at a concentration of 10 μg/mL in
PBS were placed in 24 well flat-bottom tissue culture trays (Costar), making certain to
coat the entire surface of each well. After incubating at 37° C for 2 hours or at 4°C
overnight, wells were washed 3 times with ice cold PBS followed by gentle suction
5   aspiration of well contents.

     [³H]thymidine proliferation assay. Highly pure γδ and αβ T cells were plated at
equivalent cell numbers (5,000 cells/well) in 96-well micro-titer trays, receiving either
IL-2 at 100 U/mL or PBS only. After 24 hours, sorted cells were incubated with 1 μCi
10   [³H]thymidine and after an additional 18 hours, cells were harvested onto glass fiber
filters using a standard cell harvester (PHD Cell Harvester, Cambridge, MA);
radioactivity was measured using a liquid scintillation counter. All samples were
assayed in triplicate with data presented as mean CPM.

15   *Surface staining and purification of cells by FACS.* Cultured or fresh cells were
surface stained using FITC-, PE-, or allophycocyanin (APC)-directly conjugated MAbs
recognizing CD3, CD5, TCR-γδ or TCR-αβ (Becton Dickinson, San Jose, CA).
Directly-conjugated isotype-matched irrelevant antibodies served as controls. Cells
were stained for 30 min at 4° C in staining buffer consisting of Hank's buffered saline
20   solution (HBSS, Mediatech, Herndon, VA) containing 2% FBS. Excess Ab was
removed by dilution with 10 volumes staining buffer followed by centrifugation at 500
X G. Stained cells were immediately analyzed using a FACSCalibur flow cytometer
(BDIS, San Jose, CA). For sterile sorting, stained cells were immediately sorted using
a FACS Vantage cell sorter (BDIS, San Jose, CA) equipped with a high-speed *Turbo*
25   *Sort* option. For analysis and sorting, propidium iodide (PI) uptake was used to
exclude non-viable cells during acquisition. Data analysis was performed using
CellQuest software (BDIS, San Jose, CA).

     Four color flow cytometry utilizing annexin V-FITC and PI to measure
30   *apoptosis in αβ and γδ T cell subsets.* Cultured PBMC were first simultaneously
surface stained (1 x 10⁵ total cells in 100 μL) using anti-CD3-APC and anti-TCR-γδ-
PE MAbs, as described above. Cells were subsequently washed twice with cold PBS

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and then washed twice again with 1X annexin binding buffer provided by manufacturer (Apoptosis Detection Kit, R&D Systems). Cells were then resuspended in 100 μL of binding buffer to which the appropriate volume of annexin V-FITC and PI were added, as determined by titration. Cells were incubated for 15 minutes at room temperature in

5   the dark after which time 300 μl 1X annexin binding buffer was added. Without washing, cells were immediately analyzed utilizing a dual laser, four color FACSCalibur flow cytometer (BDIS, San Jose, CA). At this point, cells were kept on ice to prevent the capping and internalization of surface-bound MAbs. Prior to acquisition of data, calibration and compensation of all fluorescence detectors (FL1 x

10   FL2; FL2 x FL3 and FL3 x FL4) was performed using cells stained with individual positive and negative control reagents in the presence or absence of annexin V-FITC and/or PI. Agonistic mouse anti-human CD95/Fas MAb CH11 (mouse IgM, Kamiya Biomedical, Seattle, WA) or mouse IgM isotype control antibody were used as positive and negative controls respectively to define apoptotic (annexin+/PI-), viable (annexin-

15   /PI-) and necrotic (annexin+/PI+) quadrants within acquisition dot-plots.

^{51}Cr release cytotoxicity assay. Melanoma cell lines SK-MEL-3, SK-MEL-5 and SK-MEL-28 (Dr. B. McAlpine, Emory University, Atlanta, GA) were utilized as targets for cytotoxicity assays. 1 x 10^6 cells were labeled with 100 μCi $Na_2^{51}CrO_4$

20   (sodium chromate, aqueous; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at 37° C and subsequently washed in RPMI containing 10% FBS. Cells were allowed to incubate an additional 30 minutes at 37° C followed by an additional wash, then plated (2 x 10^3/well) in 96 well V-bottom microtitre trays. Highly purified αβ or γδ T cells in varying numbers (final ratios, 40:1 to 0.5:1) were added to target cells in a final

25   volume of 150 μL. Trays were briefly centrifuged then incubated for 4 hours at 37° C, after which 50 μL of supernatant was removed to determine ^{51}Cr release in CPM as measured by gamma counter. Percent specific target cell lysis was calculated as [(experimental release - spontaneous release) / (maximum release - spontaneous release)] X 100. Maximum and spontaneous release were respectively determined by

30   adding either 0.1% Triton X-100 or culture medium alone to labeled target cells in the absence of effector cells. Data are presented as the mean of triplicate samples of a representative experiment.

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Mitogenic stimulation of PBMC in the presence of anti-CD2 MAb S5.2 results

in a large expansion of γδ T cells. Culture of PBMC in the presence of mitogenic anti-

CD3 MAb OKT3 and IL-2 is a well known method for the induction of in vitro T cell

proliferation, particularly αβ T cells (31-33). IL-12-dependent expansion of human

5   CD56+ αβ T cells arising in OKT3/IL-2 -stimulated PBMC cultures, particularly if

these cultures were first primed with IFN-γ 24 hours prior to stimulation with mitogens

has been described (2,3). It has since been determined that within these cultures,

monocytes serve as an important cellular source of both endogenous IL-12 and a

contact-dependent factor in the form of CD58/LFA-3, both of which are critical for the

10   *in vitro* expansion of these CD56+ αβ T cells.

In the process of examining which surface antigens played a role in CD56+ αβ T

cell expansion, monoclonal antibodies against various surface structures such as   ..

adhesion molecules or co-stimulatory receptors were separately included in PBMC

15   cultures first primed with IFN-γ, then stimulated 24 hours later with mitogenic OKT3

and IL-2. Shown in Figure 1, inclusion of one particular mouse anti-human CD2 MAb

(S5.2, IgG_{2a}), but not its isotype control, resulted in a large increase in the percentage

of γδ T cells in cultures 7 to 10 days after initiation. Several other anti-human CD2

MAbs (and their corresponding isotype controls) were assessed for their ability to cause

20   γδ T cell expansion under identical culture conditions. Neither antibody 6F10.3 (mouse

IgG_1), antibody 39C1.5 (rat IgG_{2a}) nor antibody LT-2 (mouse IgG_{2b}, not shown) were

able to significantly induce γδ T cell expansion as compared to MAb S5.2.

γδ T cell expansion induced by anti-CD2 MAb S5.2 in mitogen-stimulated

25   *PBMC cultures requires the presence of IL-12.* Mitogen-stimulated PBMC cultures

were initiated as described above, primed first with IFN-γ then stimulated 24 hours later

with mitogenic OKT3 and IL-2. After 7 to 10 days, cultures were analyzed by FACS

for the percentage of γδ T cells. Whereas anti-CD2 MAb S5.2 (but not its isotype

control) can induce γδ T cell expansion in cultures to which no exogenous IL-12 is

30   added, the addition of exogenous IL-12 to identical cultures containing MAb S5.2

results in a further increase in the percentage of γδ T cells (24% to 32%). Importantly,

IL-12 alone cannot significantly induce γδ T cell expansion in mitogen-stimulated

PBMC cultures not containing MAb S5.2 since addition of IL-12 to cultures containing only the isotype control for MAb S5.2 results in a minimal increase in γδ T cell percentage.

5   Most importantly, if a neutralizing MAb to human IL-12 (but not its isotype control) is added to mitogen-stimulated PBMC cultures initiated in the presence of MAb S5.2, γδ T cell proliferation is almost completely inhibited. These findings indicate that the S5.2-mediated γδ T cell expansion is dependent upon the presence of endogenous IL-12; however, these results also indicate that the addition of exogenous

10   IL-12 in the presence of S5.2 can further augment γδ T cell expansion in these cultures.

        S5.2-mediated, IL-12-dependent increase in γδ T cell percentage occurring in mitogen-stimulated PBMC cultures is a consequence of a preferential increase in the

15   *absolute cell numbers of γδ T cells.* By determining the fold expansion (absolute cell numbers) of both γδ and αβ T cells in mitogen-stimulated PBMC cultures, it was determined that the increase in the percentage of γδ T cells induced by the addition of S5.2 and exogenous IL-12 was not occurring at the expense of αβ T cell expansion. Cultures of fresh PBMC were initiated as described receiving IFN-γ initially (day 0),

20   followed 24 hrs later by mitogenic stimulation with OKT3 and IL-2 (day 1).On day 0, either IL-12 (10 U/mL) or PBS was added to cultures. Likewise, anti-CD2 MAb S5.2 was added at the indicated concentration (μg/mL). After 14 days, absolute numbers of both αβ and γδ T cells present in cultures were determined by multiplying the total cell number present in culture by the percentage of αβ and γδ T cells as measured by FACS.

25   Data are presented as fold expansion over starting αβ and γδ T cell number.

        In agreement with the findings above, addition of IL-12 to mitogen-stimulated PBMC in the absence of MAb S5.2 is not sufficient to induce a significant increase in the expansion of γδ T cells. Second, whereas the fold expansion of γδ T cells is greatly

30   increased by the addition of both MAb S5.2 and exogenous IL-12 (40 fold expansion to over 230 fold expansion), no corresponding increase in the fold expansion of αβ T cells is noted. Also, the addition of MAb S5.2 and IL-12 does not inhibit αβ T cell expansion

at S5.2 concentrations which promote γδ T cell expansion, indicating that a true

preferential expansion of γδ T cells is occurring in response to these stimuli. Both αβ

and γδ T cell expansion appear to be inhibited at S5.2 concentration at 10 μg/mL (or

higher). The reasons for this remain unclear.

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Anti-CD2 MAb S5.2 induces γδ T cell expansion via an agonistic and not a

blocking interaction with CD2. The existence of "accessory" or "alternate" CD2

signaling pathways triggered by MAbs to CD2 which function exclusively in γδ T cells

have previously been suggested by several investigators (28,34). While the majority of

10   anti-CD2 MAbs capable of delivering a proliferative signal to either αβ or γδ T cells

appear to do so only if combined with a second anti-CD2 MAb recognizing a separate

CD2 epitope (28,34,35), single epitope-binding anti-CD2 MAbs have been reported

which appear to stimulate only γδ T cells (28,34). In a similar manner, it is proposed

that anti-CD2 MAb S5.2 functions to induce γδ T cell expansion in heterogeneous

15   PBMC cultures. The following experiments were performed to show that MAb S5.2

functions in an agonistic and not a blocking capacity, thereby initiating rather than

inhibiting CD2 signal transduction events leading to the observed IL-12-dependent γδ T

cell expansion.

20   In mice and humans, both CD58 (LFA-3) and CD48 have each been shown to

serve as ligands for CD2; in humans, however, only CD58 has been shown to interact

with CD2 on T cells in a functionally significant manner (20, 36-38). Thus, if anti-CD2

MAb S5.2 were inducing γδ T cell expansion by *blocking* an inhibitory interaction

between CD2 and CD58, then the effect of a neutralizing anti-CD58 MAb would be the

25   same, i.e., enhancement of γδ T cell expansion. This was shown not to be the case.

Cultures of fresh PBMC were initiated as described receiving IFN-γ initially (day 0)

and OKT3 and IL-2 the next day (day 1). On day 0, either anti-CD2 MAb S5.2 (mouse

IgG$_{2a}$), mouse anti-human-CD58 MAb L066.4 (mouse IgG$_{2a}$), or mouse IgG$_{2a}$ isotype

control were added separately to identical cultures. After 14 days, cultures were

30   analyzed by FACS to determine the percentage of γδ T cells present. Under these

conditions, whereas addition of anti-CD2 MAb S5.2 resulted in the expansion of γδ T

cells, addition of a blocking MAb to CD58 did not cause the same. These data indicate

that MAb S5.2 is not causing γδ T cell expansion by disrupting a putative inhibitory CD2-CD58 interaction.

To further demonstrate that MAb S5.2 is acting in an agonistic rather than an
5   inhibitory manner, the capacity of both soluble and immobilized MAb S5.2 to induce γδ
T cell expansion within mitogen stimulated PBMC cultures was compared. Antibodies
which bind to specific cell surface receptors usually cannot trigger signal transduction
events, unless either immobilized or cross-linked. As CD14+ cells (monocytes) present
in the mitogen-stimulated cultures express FcR capable of cross-linking MAb S5.2
10   (mouse IgG$_{2a}$), the following experiments were performed using PBMC first rigorously
depleted of monocytes, as described herein.

Utilizing monocyte-depleted PBMC, cultures were initiated as described above,
stimulated on day 0 with IFN-γ, IL-12 and either soluble or plastic-immobilized MAb
15   S5.2, followed 24 hrs later by mitogenic stimulation with IL-2 and plastic-immobilized
OKT3. After 21 days, cultures were analyzed by FACS to determine the percentage of
γδ T cells present. The results of these experiments showed that γδ T cells can be
induced to expand significantly in mitogen-stimulated cultures by immobilized but not
soluble anti-human CD2 MAb S5.2. Immobilized or soluble IgG$_{2a}$ (isotype control for
20   MAb S5.2) similarly had minimal effect on γδ T cell expansion. Cultures maintained
for longer periods (up to 35 days) similarly display a preferential expansion of γδ T
cells induced by immobilized but not soluble MAb S5.2.

Enhanced γδ T cell expansion induced by MAb S5.2 does not occur simply as a
25   *consequence of increased γδ T cell proliferation.* Data derived in clones clearly show
that some anti-CD2 MAbs can preferentially induce proliferation of γδ T cells
compared to αβ T cells (34,35). If the increased γδ T cell expansion observed in S5.2-
treated cultures was occurring simply on the basis of a preferential proliferation induced
by MAb S5.2, then it is likely that γδ T cells isolated from these cultures would
30   incorporate [³H]thymidine to a greater degree than αβ T cells isolated from identical
cultures.

30

Cultures of human PBMC were initiated as described above receiving on day 0, IFN-γ, IL-12 and anti-CD2 MAb S5.2. OKT3 and IL-2 were added 24 hours later (day 1). After 24 hours (day 2), both αβ and γδ T cells were sorted to high purity from these cultures, then plated at equivalent densities (5,000 cells/well) in 96-well micro-titer

5   trays and were either stimulated with IL-2 at 100 U/mL or left unstimulated (PBS). After 24 hours (day 3), [³H]thymidine was added to cultures and 18 hours later, cells were harvested onto glass fiber filters. Data were evaluated as mean CPM of triplicate samples. These data indicate that γδ T cells isolated from S5.2-stimulated cultures do not appear to be proliferating to a greater degree than αβ T cells isolated from identical

10   cultures. This indicates that the over-representation of γδ T cells observed in longer-term S5.2-treated cultures is not only a consequence of an initial preferential proliferation induced by the anti-CD2 MAb.

IL-12-dependent MAb S5.2-mediated signaling through CD2 protects γδ T cells

15   *from activation-induced cell death.* Janssen et al. have shown that γδ T cells are exquisitely sensitive to TCR/CD3 engagement (especially in the presence of IL-2) and undergo apoptosis upon receiving mitogenic stimuli through the TCR (27). Thus, one possible interpretation of these findings is that CD2 engagement by MAb S5.2 in the presence of IL-12 provides a signal to a subset of γδ T cells which protects them from

20   activation-induced cell death (AICD) caused by mitogenic OKT3 and IL-2. It is then these apoptosis-resistant γδ T cells which eventually come to be represented in larger numbers in S5.2-stimulated cultures.

Annexin V binds with high affinity to phosphotidylserine (PS) which is

25   normally confined to the inner plasma membrane leaflet of live, non-apoptotic cells; appearance of PS on the outer plasma membrane leaflet is an early and widespread event associated with apoptosis in a variety of human cell types, regardless of the initiating stimulus. These findings have been exploited to allow the examination of apoptosis by flow cytometric means utilizing annexin V conjugated to FITC (39,40).

30   Thus, annexin V-FITC in combination with directly conjugated antibodies can be used, especially in a properly configured four color flow cytometer, to detect apoptosis

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occurring in phenotypically defined subpopulations of cells within heterogeneous cell
cultures.

5   In order to demonstrate that CD2 engagement by MAb S5.2 in the presence of
IL-12 protects γδ T cells from AICD, the following experiment was performed. By
convention, day 0 stimuli (IFN-γ, IL-12 and anti-CD2 MAb S5.2) are designated as
putative "protective" signals. PBMC cultures were initiated as described above. Those
receiving day 0 rescue signals were defined as *protected*; those receiving no day 0
signals (PBS only) were defined as *unprotected*. All cultures received OKT3 and IL-2
10   24 hours later (day 1).

     At various intervals after receiving the day 1 mitogenic signals, γδ and αβ T cell
populations within protected and unprotected cultures were simultaneously analyzed for
apoptosis using four color flow cytometry. αβ and γδ T cell populations were first
15   delineated by electronically gating on the corresponding αβ and γδ T cells defined by
anti-CD3-APC and anti-TCR-γδ-PE MAbs. Apoptosis occurring in αβ and γδ T cell
populations was then determined examining the uptake of Annexin V-FITC and PI in
the respective gated events.

20   The results of these experiments show the exquisite sensitivity to AICD
demonstrated by unprotected γδ T cells compared to protected γδ T cells. In the
absence of protective day 0 signals, mitogen stimulation induces apoptosis in a large
majority of γδ T cell with only approximately 1/3 or 36% of γδ T cells remaining viable
(Annexin-/PI-) after 2 days. In contrast, apoptosis occurs to a far lesser extent in γδ T
25   cells first protected with day 0 signals (IFN-g, IL-12 and anti-CD2 MAb S5.2). Fully
2/3 or 67% are found to be viable despite receiving the identical mitogenic stimulation.
These results also show that apoptosis occurring in αβ T cells in response to mitogenic
stimulation is negligible under these conditions. In this regard, αβ T cells both serve as
a control and support the argument that it is the γδ T cell compartment in cultures which
30   is most affected by these manipulations.

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Confirmatory experiments were next performed utilizing highly purified γδ and
αβ T cells obtained by high-speed cell sorting to exclude the likelihood that these
findings resulted from staining or compensation artifact introduced as a consequence of
complex simultaneous multi-color analysis. Using highly purified αβ and γδ T cell

5   preparations, apoptosis was measured employing Annexin V-FITC and PI staining
alone without the need for additional surface staining. PBMC cultures were initiated on
day 0 as described above. All cultures received OKT3 and IL-2 24 hours later (day 1).
After 48 hours, viable γδ and αβ T cells were sorted from both protected and
unprotected cultures; all samples immediately upon sorting were routinely >98% pure

10   and >96 % viable, as determined by PI exclusion. Sorted populations were plated at
equivalent cell densities and after 24 to 36 hours, apoptosis in each population was
determined by two color FACS employing annexin V-FITC and PI only. In agreement
with the findings above obtained in unsorted populations analyzed in four colors,
purified αβ T cells from both mitogen-stimulated protected and unprotected cultures

15   were found to be viable (Annexin-/PI-) to an equivalent degree (routinely at least 94%,
not shown). More importantly, and in complete agreement with the four color analysis
of unsorted γδ T cells, unprotected compared to protected γδ T cells were confirmed to
be far more sensitive to apoptosis induced by mitogen stimulation (54% viable versus
80% viable).

20

*Restimulation with IL-2 is a potent inducer of apoptosis in unprotected but not
protected γδ T cells.* Cultures, both unprotected and protected, can be maintained for up
to 6 to 8 weeks with weekly replenishment of tissue culture media including
recombinant human IL-2 (10-100 U/mL); during this period, cultures are not routinely

25   restimulated with other mitogens or growth factors. Despite the significant apoptosis
induced initially in unprotected γδ T cells by day 1 mitogenic stimulation (OKT3 and
IL-2), surviving γδ T cells found in these cultures at the end of the first week (prior to
refeeding) are found to be relatively viable, though still less viable than those found in
protected cultures. It should be noted, however, that even at 7 days, a significant

30   difference already exists in the percentage and absolute number of γδ T cells found in
protected versus unprotected cultures: Whereas protected cultures are comprised of up

to 25 to 30% γδ T cells, unprotected cultures almost never contain more than 7 to 10% γδ T cells at this point.

The effect of adding IL-2 to both protected and unprotected γδ T cells was also
5   demonstrated. On day 7, IL-2 (100 U/mL) was added to equivalent numbers of cells from both protected and unprotected PBMC cultures. After overnight incubation, apoptosis in αβ and γδ T cell populations was determined (day 8) by measuring the uptake of Annexin V-FITC and PI in the respective gated populations. Agonistic mouse anti-human CD95/Fas MAb CH11 (mouse IgM) or mouse IgM isotype control
10   were included in identical cultures as controls. These data indicate that while the addition of IL-2 can induce apoptosis in both protected and unprotected γδ T cells, this effect is by far more pronounced in unprotected γδ T cells where the addition of IL-2 markedly decreases the percentage of viable γδ T cells (37% viable on day 8). In contrast the addition of IL-2 only modestly decreases viability of γδ T cells in protected
15   cultures (73% viable on day 8).

Functional properties of protected γδ T cells: Cytotoxicity measured against melanoma cell lines. Recognition that MAb S5.2-mediated IL-12-dependent signals can protect human γδ T cells from mitogen-induced apoptosis provides the biological
20   basis for developing the practical means to generate large numbers of human γδ T cells The following experiment is provided to demonstrate that protected γδ T cells possess properties distinct from αβ T cells arising from within the same cultures.

Cultures of human PBMC were initiated as described above receiving on day 0,
25   IFN-γ, IL-12 and anti-CD2 MAb S5.2. OKT3 and IL-2 were added 24 hours later (day 1). After 14 days, purified populations of γδ and αβ T cells were isolated from cultures by high speed cell sorter. To avoid inadvertent activation of T cells via engagement of the T cell receptor (CD3), sorting was performed using an anti-CD5-PE MAb. For the same reason, γδ T cells were sorted as αβ TCR^-, $CD5^+$ cells. Likewise, αβ T cells were
30   sorted as γδ TCR^-, $CD5^+$ cells. The cytotoxic activity of these highly purified γδ and αβ T cells was then tested against ^{51}Cr-labeled human melanoma cell lines SK-MEL-3, SK-MEL-5 and SK-MEL-28 at various effector to target ratios. Data were evaluated as

percent specific lysis. For each of the three tumor cell lines, the percent specific lysis

by the γδ T cells exceeded that of the αβ T cells assayed. These data indicate that

apoptosis-resistant γδ T cells can mediate antitumor cytotoxicity against human

melanoma cell lines *in vitro* to a significantly greater degree than αβ T cells derived

5   from identical cultures.

EXAMPLE III

Clinical applications of the administration of the γδ T cells of this invention

to a subject and of the administration of the substances described herein for in

10   **vivo expansion of γδ T cells in a subject.**

1) Either γδ T cells expanded *ex vivo* according to the methods of this invention

or the substances described herein for increasing the percentage of γδ T cells *in vivo*

can be administered to a subject diagnosed with a malignancy, according to the dosage

15   regimens described herein, with the intent of exploiting the anti-neoplastic (anti-tumor

or anti-leukemic effect) activity of these cells. Efficacy of this treatment would be

determined by assessing a response of the malignancy as measured by tumor regression

or failure of tumor progression.

20   2) The γδ T cells expanded *ex vivo* according to the methods described herein

can be administered as an adjuvant to an allogeneic bone marrow transplant in a clinical

setting where graft failure would be a likely complication (such as where the donor is an

HLA-mismatched sibling or a matched unrelated donor). *Ex vivo* expanded donor or

recipient γδ T cells can be administered prior to, in conjunction with or following the

25   administration to the recipient of a donor bone marrow stem cell product which is first

depleted of all T cells (which is done in an attempt to minimize the likelihood of graft-

versus-host disease). The intent of including *ex vivo* expanded γδ T cells with the

transplanted cells in the recipient would be to facilitate engraftment of donor-derived

hematopoietic stem cells in the transplant recipient. Efficacy of this treatment can be

30   determined in a clinical setting by measuring a number of clinical indices as would be

well known to the clinician, such as time to full engraftment, or decreased incidence of

graft failure. Alternatively, the substances described herein can be administered to the

35

bone marrow transplant recipient according to the dosage regimens described herein for
increasing the percentage of the recipient's γδ T cells *in vivo* to impart the same
beneficial effects as described herein for the direct administration of *ex vivo* expanded
γδ T cells. Efficacy of treatment by this *in vivo* stimulation method would also be
5   assessed according to the same parameters as described herein for the administration of
ex vivo expanded γδ T cells.

3) The *ex vivo* expanded γδ T cells of this invention can be administered as an
adjuvant to standard therapy (which can include, but is not limited to, antibiotics), to a
10   subject diagnosed with an infectious process, including, but not limited to, infection
with viral pathogens (such as HIV), bacterial pathogens or other infectious agents.
Efficacy would be measured by assessing the subject's ability to effectively clear
infectious organisms according to standard protocols well known in the art.
Alternatively, the substances described herein can be administered to the subject to treat
15   an infectious process according to the dosage regimens described herein for increasing
the percentage of the recipient's γδ T cells *in vivo* to impart the same beneficial effects
as described herein for the direct administration of γδ T cells. Efficacy of treatment by
this *in vivo* stimulation method would also be assessed according to the same
parameters as described herein for the administration of γδ T cells.
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4) The *ex vivo* expanded γδ T cells of this invention can be administered as an
adjuvant to subjects diagnosed with a tissue injury, which can include but is not limited
to, tissue trauma, burns, graft-versus-host disease or autoimmune destructive processes.
γδ T cells appear to contribute to wound healing by the elaboration of a number of
25   important factors including, but not limited to, keratinocyte growth factor (KGF), also
known as FGF-7. Alternatively, the substances described herein can be administered to
the subject to treat a tissue injury according to the dosage regimens described herein for
increasing the percentage of the recipient's γδ T cells *in vivo* to impart the same
beneficial effects as described herein for the direct administration of γδ T cells.
30   Efficacy of treatment by this *in vivo* stimulation method would also be assessed
according to the same parameters as described herein for the administration of γδ T
cells.

In the protocols described above in which γδ T cells are administered to a subject, γδ T cells are first expanded *ex vivo* according to the methods described herein, typically from either an autologous or allogeneic source and delivered intravenously at a dose effective in mediating a biologically significant process, as determined according to the methods described herein. For example, the amount of cells administered to a subject can be in the range of 1×10^5 to 1×10^8 γδ T cells/kg body weight.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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What is claimed is:

1. A method of increasing the percentage of gamma-delta T cells in a population of hematolymphoid cells and which gamma-delta T cells can survive for a prolonged period, comprising:

a) contacting a population of hematolymphoid cells with interleukin 12 and a ligand of CD2 which induces responsiveness to interleukin 12; and

b) contacting the cells of step (a) with a T cell mitogen and interleukin-2.

2. The method of claim 1, wherein the hematolymphoid cells are selected from the group consisting of bone marrow cells, peripheral blood mononuclear cells and cord blood cells.

3. The method of claim 1, wherein the hematolymphoid cells are human.

4. The method of claim 1, wherein the ligand of CD2 is selected from the group consisting of an antibody which specifically binds CD2, CD58, a homologue of CD58, CD48 and a homologue of CD48.

5. The method of claim 1, wherein the T cell mitogen is selected from the group consisting of an antibody which specifically binds CD3, pokeweed mitogen, ionomycin, phorbol myristate acetate and a superantigen.

6. The method of claim 5, wherein the antibody is S5.2.

7. The method of claim 1, wherein the cells are removed from a subject and returned to the subject.

8. The method of claim 7, wherein the subject is diagnosed with a disorder selected from the group consisting of cancer, tissue injury and an infection by a pathogen.

9. The method of claim 1, wherein the cells are removed from a donor and administered to a recipient.

10. The method of claim 9, wherein the recipient is diagnosed with a disorder selected from the group consisting of graft rejection, graft-versus-host disease, cancer, tissue injury and an infection by a pathogen.

11. The method of claim 1, further comprising the step of contacting the cells of step (a) with interferon gamma.

12. The method of claim 1, further comprising the step of further increasing the percentage of γδ T cells in the population of cells of step (b).

13. The method of claim 12, wherein the percentage of γδ T cells in the population of cells is further increased by a method selected from the group consisting of fluorescence activated cell sorting, immunomagnetic separation, affinity column chromatography, cellular panning and density gradient centrifugation.

14. A method of screening a ligand of CD2 for the ability to induce responsiveness to interleukin 12 comprising:
    a) contacting a population of hematolymphoid cells with the ligand and interleukin 12;
    b) contacting the cells of step (a) with an antibody to CD3 and interleukin 2;
    c) maintaining the cells of step (b) in culture for at least seven days; and
    d) determining the percentage of viable gamma delta T cells in the population of cells of step (c), whereby greater than 10% viable gamma delta T cells identifies a ligand of CD2 having the ability to induce responsiveness to interleukin-12.

15. The method of claim 14, wherein the hematolymphoid cells are selected from the group consisting of peripheral blood mononuclear cells, bone marrow cells and cord blood cells.

16. The method of claim 14, further comprising the step of contacting the cells of step (a) with interferon gamma.

17. A population of hematolymphoid cells having greater than 10% gamma delta T cells and which gamma-delta T cells can survive for a prolonged period.

18. The population of cells of claim 17, having greater than 20% gamma-delta T cells.

19. The population of cells of claim 17, having greater than 30% gamma-delta T cells.

20. The population of cells of claim 17, having greater than 40% gamma-delta T cells

21. The population of cells of claim 17, having greater than 50% gamma-delta T cells.

22. The population of cells of claim 17, wherein the hematolymphoid cells are selected from the group consisting of peripheral blood mononuclear cells, bone marrow cells and cord blood cells.

23. A method of treating cancer in a subject comprising administering to a subject diagnosed with cancer an effective amount of the cells of claim 17.

24. A method of treating an infection in a subject comprising administering to a subject diagnosed with an infection an effective amount of the cells of claim 17.

25. A method of promoting wound healing in a subject comprising administering to a subject having a wound an effective amount of the cells of claim 17.

26. A method of enhancing bone marrow engraftment in a bone marrow recipient comprising administering to the recipient an effective amount of the cells of claim 17.

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PARDOUX et al. Functional Interaction Between TGF-beta and IL-12 in Human Primary Allogeneic Cytotoxicity and Proliferative Response. The Journal of Immunology. 1997, Vol.158, No.1, pages 136-143.	1-26
A	MARX et al. Activation of Human gamma-delta T Cells by Mycobacterium tuberculosis and Daudi Lymphoma Cells. The Journal of Immunology. 1997, Vol. 158, No. 6, pages 2842-2848.	1-26
A	WESCH et al. Mycobacteria-reactive gamma-delta T cells in HIV-infected individuals: lack of Vgamma9 cell responsiveness is due to deficiency of antigen-specific CD4 T helper type 1 cells. Eur. J. Immunol. 1996, Vol. 26, No. 3, pages 557-562.	1-26

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 MAY 1999	22 JUN 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PREMA MERTZ
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UETA et al. Interleukin-12 activates human gamma-delta T cells: synergistic effect of tumor necrosis factor-alpha. Eur. J. Immunol. 1996, Vol. 26, No.12, pages 3066-3073.	1-26
A	SKEEN et al. Activation of gamma-delta T Cells for Production of IFN-gamma Is Mediated by Bacteria Via Macrophage-Derived Cytokines IL-1 and IL-12. The Journal of Immunology. 1995, Vol. 154, No. 11, pages 5832-5841.	1-26
A	KLEIN et al. Herpesvirus saimiri Immortalized gamma-delta T Cell Line Activated by IL-12. The Journal of Immunology. 1996, Vol. 156, No. 8, pages 2754-2760.	17-26
A	SATOH et al. Cytotoxic gamma-delta or alpha-beta T Cells with a Natural Killer Cell Marker, CD56, Induced from Human Peripheral Blood Lymphocytes by a Combination of IL-12 and IL-2. The Journal of Immunology. 1996, Vol. 157, No. 9, pages 3886-3892.	1-26

INTERNATIONAL SEARCH REPORT	International application No.
	PCT/US99/05355

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

    Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                         ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/00, 5/06, 5/08; A61K 38/19, 38/20, 38/21, 49/00; G01N 33/53, 33/567

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/2, 8, 12, 885; 424/85.1, 85.2, 85.5, 184.1, 198.1; 435/375, 325, 372, 372.1, 372.3, 373, 374, 7.1, 7.2, 7.21, 7.24

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

514/2, 8, 12, 885; 424/85.1, 85.2, 85.5, 184.1, 198.1; 435/375, 325, 372, 372.1, 372.3, 373, 374, 7.1, 7.2, 7.21, 7.24

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, BIOSIS, EMBASE
search terms: gamma-delta T cells, interleukin-12, interleukin-2, T cell mitogen, CD2 ligand, interferon gamma, screening, CD3 antibody, treatment, administration, therapy, method

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-13, drawn to a method of increasing the percentage of gamma-delta T cells in a population of hematolymphoid cells comprising contacting the cells with IL-12 and a ligand of CD2 and then with a T cell mitogen and IL-2.
Group II, claims 14-16, drawn to a method of screening a ligand of CD2 for the ability to induce responsiveness to IL-12.
Group III, claims 17-22, drawn to a population of hematolymphoid cells having 10% gamma-delta T cells which gamma-delta T cells can survive for a prolonged period.
Group IV, claims 23-26, drawn to a method of treating cancer comprising administering a population of hematolymphoid cells having 10% gamma-delta T cells which gamma-delta T cells can survive for a prolonged period.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first-recited method, a method of increasing the percentage of gamma-delta T cells in a population of hematolymphoid cells comprising contacting the cells with IL-12 and a ligand of CD2 and then with a T cell mitogen and IL-2. Further pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.